

Susceptibility of Dipel-Resistant and -Susceptible *Ostrinia nubilalis* (Lepidoptera: Crambidae) to Individual *Bacillus thuringiensis* Protoxins

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ABSTRACT Dipel-resistant and -susceptible strains of *Ostrinia nubilalis* (Hübner) were evaluated for larval mortality and growth inhibition when fed diets containing individual *Bacillus thuringiensis* protoxins. Resistance ratios for four of the protoxins in Dipel (Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa) were 170-, 205-, 524-, and >640-fold, respectively, considerably higher than the 47-fold resistance to Dipel. The Dipel-resistant strain was 36-fold resistant to Cry1Ba, a protoxin not present in Dipel. Another non-Dipel protoxin, Cry1Ca, did not cause significant mortality for either resistant or susceptible larvae with doses as high as 1.0 mg/ml. In an evaluation of larval growth inhibition, resistance to Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Ba was significant at concentrations of 0.054 and 0.162 $\mu\text{g}/\text{ml}$. However, growth inhibition with Cry2Aa was not significant at either dose. These data provide information on the spectrum of resistance and cross-resistance to individual Cry protoxins in this strain.

KEY WORDS Cry protoxins, Bt resistance management, cross-resistance, European corn borer, Bt toxicity

CRYSTALLINE (CRY) PROTEINS PRODUCED by *Bacillus thuringiensis* Berliner (Bt) are toxic to several economically important insect pests, particularly those in the order Lepidoptera (Höfte and Whitely 1989, Widner and Whiteley 1989, Schnepf et al. 1998). Several commercial insecticidal formulations, such as Dipel ES (Valent BioSciences Co. Libertyville, IL) and Javelin (Thermo Trilogy, Columbia, MD), have been developed from different Bt strains to be used as biological insecticides against insect pests. More recently, the genes controlling expression of these toxins also have been inserted into the genome of certain crop plants to render them resistant to insect pests (Perlak et al. 1990, Adang et al. 1993, Koziel et al. 1993).

The European corn borer, *Ostrinia nubilalis* (Hübner), is one of the most damaging pests of corn, *Zea mays* L., in North America and has been one of the targets for commercial applications of Bt insecticidal formulations (McWhorter et al. 1972, Mason et al. 1996, Sloderbeck et al. 2004). This insect also has been the primary target for transgenic Bt corn hybrids. The long-term durability of Bt toxins is threatened for both applications if insects develop resistance to Cry pro-

teins (McGaughey 1985, Gould et al. 1992, Tabashnik 1994). It is therefore important to understand the mechanisms of resistance to Cry toxins in laboratory-developed resistant strains so that sound resistance management strategies can be developed that are based on an understanding of the physiological mechanisms likely to develop in the field.

The KS-SC strain of *O. nubilalis* is ≈ 70 -fold resistant to Dipel ES, one of the highest levels of resistance to a Bt formulation that has been documented for this insect (Huang et al. 1997, 1999b; Bolin et al. 1999, Chaufaux et al. 2001, Siqueira et al. 2004). This strain was obtained by exposing larvae to low concentrations of Dipel ES (*B. thuringiensis* subsp. *kurstaki* HD-1) (Huang et al. 1997). Resistance in this strain is inherited as an incompletely dominant trait (Huang et al. 1999a) with moderate levels of heritability (Huang et al. 1999b). Resistance has been associated with reduced digestive proteinase activity resulting in reduced protoxin activation (Huang et al. 1999c; Li et al. 2004a, 2005). Although resistant larvae had a significant level of resistance to Dipel ES, they were not able to survive on high-expression Bt corn plants (expressing Cry1Ab) in greenhouse trials (Huang et al. 2002).

The Dipel ES formulation contains a complex mixture of spores, protoxins, and other formulation products (Masson et al. 1989). Cry protoxins found in Dipel ES include Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B. In general, Cry1A protoxins are toxic to various lepidopterans, whereas Cry2 proteins are toxic to lepidopterans and dipterans. Resistance that devel-

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ops in response to selection with a protoxin mixture, such as Dipel, can have a complex pattern of resistance and cross-resistance to individual Cry proteins (Gould et al. 1992, Tabashnik et al. 1993, McGaughey and Oppert 1998). At the time of this study, no information was available on the sensitivity of the KS-SC Dipel-resistant strain to the individual protoxins found in Dipel or to protoxins not found in Dipel. In the current study, the sensitivity of the KS-SC Dipel-resistant strain to six individual protoxins, including four of the five protoxins found in Dipel and two protoxins not found in Dipel, was determined by larval mortality and larval growth inhibition bioassays.

Materials and Methods

Insects. The Dipel-resistant strain (KS-SC) of *O. nubilalis* was derived from 49 egg masses collected in cornfields near St. John, KS (Huang et al. 1997). Neonates from this field-collected strain were reared on a meridic diet (Reed et al. 1972). The Dipel-resistant strain was developed by exposing neonates to diet containing Dipel ES (Abbott Labs, Chicago, IL) at doses that resulted in 80–95% mortality. After seven generations of selection, resistance to Dipel ES increased to 70-fold (Huang et al. 1997). The level of resistance in this strain has not increased with continued selection (Huang et al. 1999b). At the time of bioassays for this study, the susceptible strain had been reared in the laboratory for >45 generations, and the resistant strain had been selected for >40 generations.

Sources of Bt Cry Protoxins and Native *Escherichia coli* Proteins. Four Dipel component (Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa) and two non-Dipel component (Cry1Ca and Cry1Ba) protoxins were evaluated in this study. Five recombinant and two parental nonrecombinant *E. coli* cultures and one *B. thuringiensis* culture were obtained from the *Bacillus* Genetic Stock Center, The Ohio State University, Columbus, OH. The recombinant cultures: toxins were ECE52:Cry1Aa, ECE54:Cry1Ab, ECE53:Cry1Ac, ECE125:Cry1Ca, and ECE126:Cry2Aa. The nonrecombinant ECE73 (or JM103) was the parental culture of ECE52, ECE53, and ECE54, and the nonrecombinant DH5 α was the parental culture of ECE125 and ECE126 (Zeigler 1999). Individual protoxins were isolated from recombinant *E. coli* cultures by using a method described by Ge et al. (1990). Protein samples also were extracted from the two parental *E. coli* cultures, ECE73 and DH5 α , with the same procedures used to extract Cry protoxins from recombinant *E. coli*. The protoxin Cry1Ba was isolated from *B. thuringiensis* HD-2. The HD-2 isolate was grown at 30°C for 48 h in CCY medium (Stewart et al. 1981). Spores and crystals were collected by centrifugation at 9,700 \times *g* at 4°C for 10 min. Pellets were washed four times with 1 M NaCl, 10 mM EDTA and resuspended in deionized water. The relative amount of Cry protoxin in each sample was determined by densitometric analysis of the protoxin band compared with a bovine serum albumin standard (BSA) resolved by sodium dodecyl sulfate-polyacrylamide gel elec-

trophoresis (Laemmli 1970), by using both Coomassie-stained gels and Western blot analysis as described by Li et al. (2004a) with imaging by an Odyssey infrared imaging system (LI-COR, Lincoln, NE) (data not shown). Total protein concentrations were determined by the Bradford method (Bradford 1976) by using the Coomassie Plus protein assay (Pierce Chemical, Rockford, IL) with BSA as a standard.

Larval Mortality Bioassays. The bioassay procedures were similar to those previously described by Huang et al. (1997). Individual Bt protoxins and controls (native *E. coli* proteins or deionized water) were suspended and diluted in deionized water containing 0.1% (vol:vol) Triton X-100. According to gel analysis, the amount of Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cry1Ba, and Cry1Ca protoxin in each suspension was 66, 51, 29, 42, 50, and 18%, respectively. The amount of protoxin used in the bioassay was adjusted according to these percentages. Based on preliminary tests, each Bt protoxin or native *E. coli* protein was assayed using five concentrations that were selected to produce a broad range of mortalities to facilitate probit analysis. The concentration ranges of Bt protoxins for the susceptible strain were 1.5–79 μ g/ml for Cry1Aa, 1.0–24 μ g/ml for Cry1Ab and Cry1Ac, 0.5–90 μ g/ml for Cry2Aa, 0.4–26 μ g/ml for Cry1Ba, and 10–2,700 μ g/ml for Cry1Ca. The concentration range of Bt protoxins for the resistant strain was 40–2,700 μ g/ml for Cry1Aa, 20–2,600 μ g/ml for Cry1Ab, 65–2,400 μ g/ml for Cry1Ac, 30–4,900 μ g/ml for Cry2Aa, 8.0–430 μ g/ml for Cry1Ba, and 10–2,700 μ g/ml for Cry1Ca. Dipel (1.69×10^7 IU/ml) was assayed at concentrations of 0.03–2.43 ml/kg for the susceptible strain and 0.27–21.87 ml/kg for the resistant strain to evaluate changes in resistance under continuous selection (Huang et al. 1997, 1999b).

Twenty-two milliliters of artificial diet was added to 2 ml of diluted *E. coli* protein or Bt protoxin suspensions and vigorously mixed with a syringe. Approximately 0.5 ml of the diet was dispensed into each cell of a 128-cell bioassay tray (Bio-Ba-128, CD International, Pitman, NJ). After diet solidification, one neonate (<24 h old) was placed on the diet surface within each cell, and the cells were sealed with perforated plastic covers (Bio-CV-16, CD International). Bioassay trays were placed in a growth chamber maintained at 25°C with a photoperiod of 16:8 (L:D) h and 60% RH. Larval mortality was recorded on the seventh day after insect inoculation. There were three to four replications for each concentration of the *E. coli* proteins or Bt protoxins, and each replication included 16 neonates.

Larval Growth Bioassays. The procedure for larval growth bioassays was modified slightly from that described for larval mortality bioassays. The native *E. coli* proteins or individual Cry protoxins were first solubilized in 20 mM Na₂CO₃, pH 9.6, for precise measurement and dilution of the concentrations. The series of concentrations were prepared by serial dilution in the same buffer. The *E. coli* protein and Bt protoxin concentrations used in the larval growth assays were lower than those in the larval mortality assays, at

Table 1. Effect of *B. thuringiensis* Cry protoxins on larval survival of Dipel-resistant and -susceptible strains of *O. nubilalis*

Protoxin	Insect strain	Slope \pm SE	LC ₅₀ (95% CI) ^a (μ g/ml) ^b	χ^2 ^c	df	Resistance ratio (95% CI) ^d
Cry1Aa	Susceptible	1.18 \pm 0.24	4.59 (2.52-7.25)	9.97	14	170 (82.3-350)
	Resistant	0.82 \pm 0.14	779 (448-1680)	14.2	13	
Cry1Ab	Susceptible	1.06 \pm 0.18	4.60 (2.79-7.07)	14.3	13	205 (81.9-514)
	Resistant	0.54 \pm 0.11	944 (408-4,130)	15.0	13	
Cry1Ac	Susceptible	1.34 \pm 0.35	3.48 (1.98-5.72)	6.43	13	524 (242-1,140)
	Resistant	1.29 \pm 0.30	1,820 (1,110-4,630)	4.3	13	
Cry2Aa	Susceptible	1.98 \pm 0.30	7.73 (5.48-12.6)	18.1	13	>640
	Resistant	n/a ^e	>4,900 ^f	n/a	13	
Cry1Ba	Susceptible	1.87 \pm 0.28	2.52 (1.72-3.62)	14.0	14	36.2 (19.1-69.0)
	Resistant	1.15 \pm 0.24	91.3 (56.4-191)	12.0	14	
Dipel	Susceptible	1.65 \pm 0.18	0.19 (0.13-0.28)	20.8	13	47.3 (32.4-69.1)
	Resistant	2.20 \pm 0.28	9.05 (6.90-12.40)	13.8	13	

^a LC₅₀ represents the concentration of protoxin resulting in 50% mortality of first instars of *O. nubilalis* larvae.

^b Unit of LC₅₀ for Dipel is milliliters per kilogram of diet.

^c No χ^2 values were significant at $P = 0.05$ level, which means that all mortality-dose linear models significantly fit the probit-dose model.

^d Resistance ratios were calculated using POLO PC software based on LC₅₀.

^e Data not available.

^f Mortality at the dose was 8.3%.

concentrations that inhibited larval growth but without significant mortality. The concentration of proteins from the two parental *E. coli* cultures ranged from 5 to 25 μ g/ml. The ranges of Bt protoxin concentrations for the susceptible strain were 0.270-35.20 μ g/ml for Cry1Ca, 0.002-4.370 μ g/ml for Cry1Ba, and 0.002-0.162 μ g/ml for the other four protoxins. The concentration ranges for the resistant strain were 0.730-88.00 μ g/ml for Cry1Ca and 0.054-4.370 μ g/ml for the other five protoxins. The buffer (100 μ l) was incorporated into 24 ml of diet for the control, and bioassays were conducted as described previously, except that there were four replications for each of the *E. coli* proteins or Bt protoxins and eight larvae were assayed in each replication. Larval weights were determined on the seventh day after inoculation.

Data Analysis. Larval mortality data were corrected using the method described by Abbott (Abbott 1925) and were analyzed by probit analysis (Finney 1971) by using the POLO-PC statistical software (LeOra Software 1987) to provide LC₅₀ values, 95% confidence intervals (CI), and slopes of the dose-mortality curves. Resistance ratios were calculated by POLO-PC and were considered significant when the 95% CI did not include the value 1 (Robertson and Preisler 1992).

Larval growth inhibition was examined using probit analysis to calculate the EC₅₀ (effective concentration of Cry protoxin that resulted in 50% growth inhibition relative to the larvae reared on the control diet) for each protoxin. However, the relative potency 95% CI that was calculated based on the EC₅₀ was undefined, because the doses did not adequately cover the growth inhibition range to facilitate probit analysis. There was also considerable variation in larval weights at the lower doses. Therefore, to compare the relative growth inhibition, data for two concentrations that were common across strain and protoxin (0.054 and 0.162 μ g/ml) were analyzed using a two-way analysis of variance (ANOVA) (SAS Institute 1990) with the insect strain (resistant and susceptible) and protoxin concentration (0.054 and 0.162 μ g/ml) as the two

main factors. Growth inhibition for Cry1Ca was analyzed by one-way ANOVA for all doses across each strain because there were no common doses between the two strains.

Results

Larval Mortality. Native *E. coli* proteins from parental cultures DH5 α or ECE73 did not significantly affect the survival of either Dipel-resistant (KS-SC) or -susceptible larvae, even at concentrations as high as 1,500 μ g/ml. The highest mortalities in the bioassay were 5.5% for the resistant strain and 11.1% for the susceptible strain. In most cases, there was no mortality.

The LC₅₀ values for the resistant strain were significantly higher than those for the susceptible strain for five of the six protoxins, and also for Dipel (Table 1). Resistance ratios for four of the Cry protoxins found in Dipel, Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa were 170-, 205-, 524-, and >640-fold, respectively. The Dipel-resistant strain was also 36.2-fold resistant to Cry1Ba and 47.3-fold resistant to Dipel. Neither strain was very sensitive to the Cry1Ca protoxin, and only at the highest tested concentration (2,680 μ g/ml) was there a difference in susceptibility of the two strains. At this concentration, the mortality of the susceptible and resistant strains was 43 and 8%, respectively.

Larval Growth. Compared with larvae reared on the untreated control diet, native *E. coli* proteins extracted from the two parental *E. coli* cultures did not significantly alter the growth of either resistant or susceptible *O. nubilalis* larvae at concentrations up to 17 and 25 μ g/ml for DH5 α and ECE73, respectively.

In a two-way ANOVA analysis of growth inhibition of resistant and susceptible *O. nubilalis*, the main effect of strain was significant for all five of the protoxins that were tested at 0.054 and 0.162 μ g/ml (Table 2). At the 0.054 μ g/ml dose, the mean growth inhibition was significantly higher for the susceptible strain than

Table 2. Mean (±SE) larval growth inhibition for resistant (R) and susceptible (S) *O. nubilalis* exposed to two concentrations of *B. thuringiensis* Cry protoxins

Concn. (µg/ml)	Cry1Aa		Cry1Ab		Cry1Ac		Cry2Aa		Cry1Ba	
	0.054	0.162	0.054	0.162	0.054	0.162	0.054	0.162	0.054	0.162
R-strain mean %	45.8b (±8.77)	57.6b (±6.39)	58.4b (±2.14)	90.0a (±0.96)	50.0c (±1.84)	60.5b (±1.41)	30.0b (±8.22)	52.4ab (±5.53)	24.8c (±4.25)	36.1bc (±5.98)
S-strain mean %	95.7a (±0.18)	97.2a (±1.02)	91.3a (±2.95)	94.0a (±2.80)	91.4a (±3.48)	90.1a (±3.22)	55.0ab (±9.98)	66.2a (±7.52)	51.8b (±5.79)	76.2a (±5.28)
ANOVA										
Concentration, df = 1	F = 2.110	P = 0.177	F = 53.64	P < 0.001	F = 3.120	P = 0.103	F = 4.350	P = 0.061	F = 11.13	P = 0.006
Strain, df = 1	F = 95.50	P < 0.001	F = 61.55	P < 0.001	F = 180.7	P < 0.001	F = 5.790	P = 0.035	F = 39.09	P < 0.001
Interaction, df = 1	F = 1.290	P = 0.282	F = 37.86	P < 0.001	F = 4.980	P = 0.046	F = 0.480	P = 0.502	F = 1.500	P = 0.244

Means for the same protoxin with the same letter are not significantly different (P = 0.05, least significant difference).

it was for the resistant strain for all except Cry2Aa protoxin. At 0.162 µg/ml, the growth inhibition was significantly higher only for Cry1Aa, Cry1Ac, and Cry1Ba (Table 2). The interaction between strain and concentration was significant for Cry1Ab and Cry1Ac. For Cry1Ab, growth inhibition at 0.162 µg/ml was >90% for both strains, and for Cry1Ac at both doses >90% for the susceptible strain (Table 2; Fig. 1). Although the main effect of strain on growth inhibition was significant for Cry2Aa (P = 0.04), there was no significant difference in growth inhibition of resistant and susceptible larvae at either dose (Table 2). Growth inhibition with Cry1Ca was significant at all doses for the susceptible strain (P < 0.01) but was significant for the resistant strain only at doses >44 µg/ml (Fig. 1). At lower doses of Cry1Ca (0.7–11 µg/ml), the growth of resistant larvae was stimulated by 47–25%. Growth inhibition was more significant for susceptible than for resistant larvae (P < 0.01).

Discussion

Native *E. coli* proteins extracted from the parental isolates did not cause significant mortality or growth inhibition for either Dipel-resistant or -susceptible strains of *O. nubilalis*. This was an indication that the larval mortality and growth inhibition observed in the protoxin bioassays was attributed to the toxicity of the respective Cry protoxins.

The fact that the Dipel-resistant strain had significant resistance to several individual Bt protoxins in mortality bioassays was not unexpected, because the strain was selected with Dipel, which contains a mixture of Cry protoxins. These data demonstrate that the Dipel-resistant strain of *O. nubilalis* was resistant to at least four of the five protoxins found in Dipel. The fifth Dipel protoxin, Cry2B, was not available for analysis. The Dipel-selected *O. nubilalis* strain also was 36-fold resistant to Cry1Ba. This Cry protoxin is not found in Dipel, demonstrating cross-resistance. The identity of amino acid sequences between Cry1A and Cry1B is low (≈55%) (Schnepf et al. 1998). The relatively low resistance ratio to Cry1Ba in mortality bioassays could be because of interactions between spores and Cry protoxins, because *B. thuringiensis* HD-2, from which we obtained Cry1Ba, produces spores that have not been eliminated during our purification process.

Selection with formulated spore-crystal mixtures, such as Dipel, also has produced complex resistance patterns in other insects (reviewed by Ferré and Van Rie 2002). For example, a Dipel-selected Indianmeal moth strain, *Plodia interpunctella* (Hübner), was 250-fold resistant to Dipel (McGaughey and Beeman 1988), whereas it was >800-fold resistant to Cry1Ab. However, this Dipel-resistant strain was nearly four-fold more susceptible to Cry1Ca (Van Rie et al. 1990). The diamondback moth, *Plutella xylostella* (L.), is the only insect species that has evolved high levels of resistance to Bt sprays in the field (Tabashnik et al. 1990). This Bt-resistant *P. xylostella* strain (NO-QA) was exposed to additional laboratory selection with

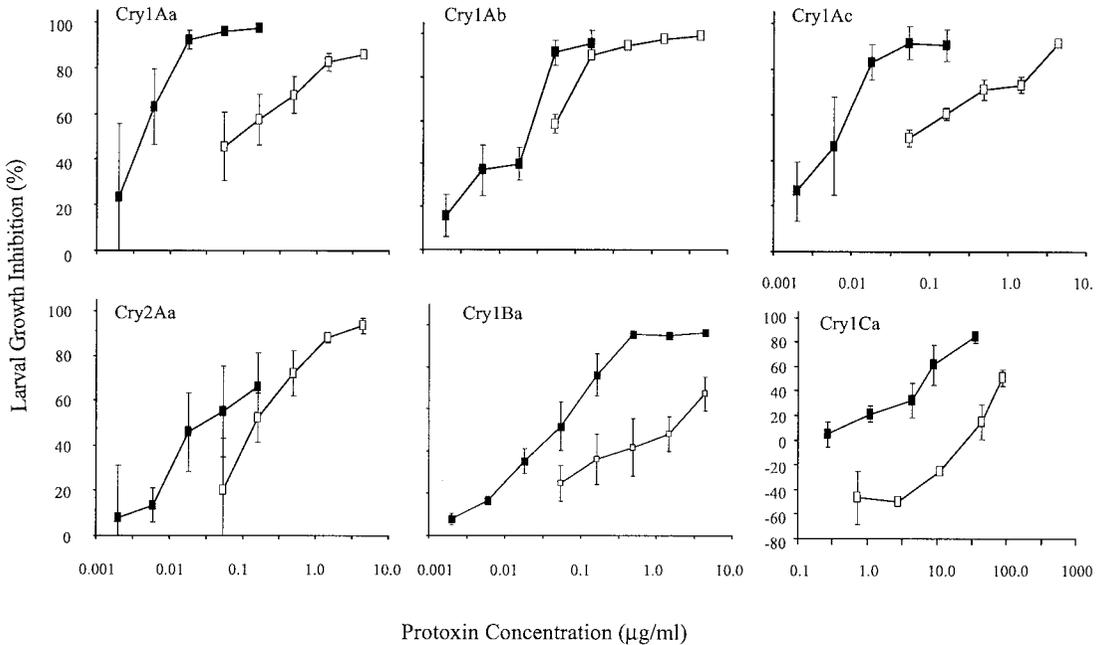


Fig. 1. Effect of *B. thuringiensis* Cry protoxins on the growth of Dipel-resistant (empty square) and -susceptible (solid square) larvae of *O. nubilalis*. Data represent the means of four replicates ($n = 32$). Error bars represent standard deviations.

Dipel, and resistance increased rapidly to >1,000-fold (Tabashnik et al. 1991, 1993). Dipel-resistant *P. xylostella* displayed high levels of resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja, but not to Cry1Ba, Cry1Bb, Cry1Ca, Cry1Da, Cry1Ia, and Cry2Aa. Tang et al. (1996, 1999) reported a *P. xylostella* strain that had 1,500-fold resistance to Javelin, another commercial formulation of Bt species *kurstaki* (NRD12), and grew well on noncommercial transgenic broccoli expressing Cry1Ac.

In our Dipel-resistant *O. nubilalis* strain, Cry1Ab and Cry1Ac share binding sites in the gut membrane (Li et al. 2004b). However, resistance to Cry1Ab and Cry1Ac was not associated with altered receptor binding in this strain. It is unknown whether Cry2Aa and Cry1Ba have binding sites different from those for Cry1A toxins in *O. nubilalis*, but the likelihood of shared binding sites of these toxins is low (English et al. 1994, Fiuza et al. 1996). It is also unknown whether the high-level resistance (>640-fold) to Cry2Aa in our resistant strain is associated with altered receptor binding. Cry1Ca did not cause significant mortality to either strain of *O. nubilalis*. This result supports our previous finding that activated Cry1Ca does not bind to the brush border membrane vesicles from either resistant or susceptible larvae of *O. nubilalis* (Li et al. 2004b).

However, resistance in the Dipel-resistant strain of *O. nubilalis* was associated with reduced protoxin activation (Huang et al. 1999c; Li et al. 2004a, 2005). Alteration of a common step in the mode of action, protoxin solubilization and/or activation, could lead to cross-resistance among many protoxins. Dipel-resis-

tant *O. nubilalis* have a significantly lower soluble trypsin-like activity (Huang et al. 1999c, Li et al. 2004a). The reduced trypsin-like proteinase activity may be associated with a general Cry protein resistance in this strain, because trypsin-like proteinases are required in both the solubilization and activation of protoxins in insect guts (Oppert 1999).

The differences in resistance ratios of protoxins may be related to differences in the relative concentrations of individual Dipel component protoxins (Masson et al. 1989, Liu et al. 1996). However, differences also may be associated with the mode of action of the respective protoxins. For example, the mode of action of Cry2Aa has been proposed to be different from Cry1A toxins (English et al. 1994). In the current study, Cry2Aa resistance in the resistant strain was considerably higher (>640-fold) than to the other protoxins (170- to 524-fold). The primary sequence of Cry2A is different from other Cry proteins (Schnepf et al. 1998), and Cry2A proteins are relatively insoluble in the gut of lepidopteran insects (English et al. 1994). Therefore, insolubility coupled with lower proteinase activity could contribute to the higher resistance ratio to Cry2Aa in the Dipel-resistant strain.

Although resistance to Cry2Aa was the highest among the tested protoxins in mortality bioassays, it was not significant at concentrations of 0.054 and 0.162 µg/ml in the growth bioassays. The differences in resistance in the two bioassays may indicate that growth inhibition bioassays are less sensitive than mortality bioassays. Differences in resistance in the two bioassays also may be associated with the preparation of protoxins. In the larval growth evaluations,

the protoxins were presolubilized by alkaline pH before bioassay. In the mortality bioassays, the protoxins were not solubilized, and insects with lower serine proteinase activity may be less sensitive.

Differences in larval growth inhibition of the two strains were significant when analyzed at selected doses of most protoxins. Resistance ratios calculated by probit analysis from larval growth bioassays were not statistically different because of protoxin concentration ranges that resulted in large or undefined 95% CIs. The lowest tested doses for the resistant strain resulted in >50% inhibition for some of the protoxins, which resulted in an undefined 95% CI for the EC₅₀ resistance ratio, even though the EC₅₀ 95% CI were reasonably narrow. Another problem was the considerable variability in some of the larval weights, particularly for the lower doses in bioassays with each strain. Growth inhibition bioassays can be improved when dose ranges include doses that bracket the EC₅₀, as well as including more replicates at each dose.

Regardless, the difference in the effect of protoxin in the two *O. nubilalis* strains was less in larval growth bioassays than in mortality bioassays. Dipel-resistant larvae completed development on nontreated diet after neonates were exposed for 10–15 d to a diet containing Dipel, because larvae cannot survive and develop continuously on Dipel-treated diet. In other Bt-resistant insects, such as pink bollworm, *Pectinophora gossypiella* (Saunders) (Tabashnik et al. 2000); tobacco budworm, *Heliothis virescens* (F.) (Gould et al. 1995); and *P. xylostella* (Tang et al. 1999, Zhao et al. 2000), resistant larvae were reared entirely on diets or transgenic Bt plants containing Cry proteins. The high levels of resistance to Bt in these insects have been associated with alterations in receptor binding (Ferré et al. 1991, Lee et al. 1995, Zhao et al. 2000, González-Cabrera et al. 2003, Morin et al. 2003). However, other Bt-resistant strains of *O. nubilalis* also were unable to complete development on Bt transgenic corn (Siqueira et al. 2004). Furthermore, many of the resistance ratios based on mortality or growth inhibition were lower than in our study, but these resistance ratios often did not include confidence intervals (Chaufaux et al. 2001, Siqueira et al. 2004). Differences in statistical analysis make it difficult to relate the significance of previous findings to our study. Comparisons of LC₅₀ and EC₅₀ values in other resistant insects are needed to better understand the relevance to resistance, and it is important to use correct dose ranges, increased sample sizes, and appropriate statistical methods to determine significance for these comparisons to be meaningful.

One of the problems in comparisons of susceptibility is the considerable variation in the baseline response of *O. nubilalis* to Bt toxins. Bolin et al. (1999) found a 2.7-fold difference in the LC₅₀ of Cry1Ac in bioassays with *O. nubilalis* larvae performed at different times. Hua et al. (2001) reported that the LC₅₀ of Cry1Ab and Cry1Ac protoxins were 0.29 and 0.08 µg/ml for *O. nubilalis*, 16- and 44-fold lower, respectively, than those of our susceptible strain. Differences also have been reported in Cry1Ab protoxin

susceptibility in other field and laboratory strains (Siegfried et al. 1995, Marçon et al. 1999). The variation in susceptibility of an insect strain to a Cry protein assayed at different times or by different laboratories may be because of factors such as different geographical populations, bioassay conditions (vitality of insects, temperature, relative humidity, exposure duration), and bioassay methods (e.g., surface overlay or diet incorporation). In our laboratory, bioassays are conducted using diet-incorporated toxins. The LC₅₀ of the susceptible strain has been consistent over the past 7 yr (≈0.07–0.17 ml/kg) and the resistant strain has remained constant in its resistance level to Dipel.

Bt formulations such as Dipel have been available for farmers to use in controlling *O. nubilalis* for many years (McWhorter et al. 1972), but they have not been widely employed. Even today, with the widespread commercial production of transgenic Bt corn, many state insect management recommendation guides list sprayable Bt formulations as an option for managing corn borer (Mason et al. 1996, Sloderbeck et al. 2004). With some sectors of the public expressing increasing concern over food safety and chemical insecticide residue, organic farming could become more significant. Thus, it is important to retain the effectiveness of environmentally benign Bt microbial insecticides. Our results indicate that the laboratory-selected *O. nubilalis* strain has developed resistance to several individual Bt protoxins after repeated exposure to Dipel. If Dipel or other protoxin-based Bt formulations become popular and are used extensively against *O. nubilalis*, resistance may develop to one or more of the individual Bt toxins.

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